Specific IgE Antibody Responses to Somatic and Excretory-Secretory Antigens of Third Stage *G. spinigerum* Larvae in Human Gnathostomiasis

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Abstract

Specific IgE antibody levels in the serum of patients with proven gnathostomiasis and in those with intermittent cutaneous migratory swelling (CMS) were determined by the enzyme-linked immunosorbent assay (ELISA) using somatic extract and excretory-secretory (ES) products of *Gnathostoma spinigerum* infective larvae as antigens. The third stage larval used were obtained from naturally infected eels. There was an increase in specific IgE antibody to both antigens in these patients. The mean levels of these specific IgE antibodies were significantly higher than that of the healthy control ($P<0.01$). Comparison between using somatic extract and ES products in the test showed, a positive result in the group of suspected patients with gnathostomiasis or CMS was significantly higher when using ES products (81.81%) than somatic extract (59.09%) as the antigens ($P<0.05$). However, both somatic and ES antigens cross-reacted with other parasitic sera. The overall sensitivity of the ELISA for these IgE antibodies detection were 71.87 per cent and 87.50 per cent with somatic and ES antigens, respectively. The specificity was 57.53 per cent when somatic antigen was used and increased to 69.86 per cent when ES antigen was used. The positive and negative predictive values of the test were 42.59 per cent and 82.35 per cent by using somatic antigen. Both of these values, were also increased to 56.00 per cent and 92.72 per cent by using the ES antigen. It is obvious that more potential components may be present in ES products than those in the somatic extract. The ES antigen may have to be further purified and may be suitable for evaluation of the effectiveness of chemotherapy. As such, the antibody responses to secreted products are more closely related to active infection than the anti-whole worm antibody that may persist following the death of the parasites. However, in this disease, the effect of the IgE antibody on its pathophysiology it is still not known.

Key word : IgE Antibody, *Gnathostoma spinigerum*, Somatic Antigen, Excretory-Secretory Antigen

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For immunological studies, gnathostomiasis has received less attention in antibodies studies than other parasitic infections, for example; trichinellosis, toxocariasis and anisakiasis etc[1,2]. Many nematode infections elicit pronounced antibody responses, involving all major isotypes[3,4]. An increase in parasite-specific IgE antibody is a particular feature of those having a tissue migration[3, 5].

Gnathostoma spinigerum is a nematode in dogs and cats but can cause severe and even fatal infection in humans[6-8]. Man is an accidental host and acquires the infection by eating inadequately processed intermediate hosts, e.g. fresh water fish, frogs, and snakes which contain encapsulated third stage larvae (L_3s). Following ingestion, the parasite may migrate to any tissue of the body. The most common outcome of the disease in humans is intermittent cutaneous migratory swelling[9]. The parasite migration within the central nervous system or eye can result in intracranial haemorrhage, eosinophilic meningoencephalitis, or blindness[7-9]. The patients can die if they are not properly diagnosed and managed. The disease is fairly common in Thailand and has also been reported from Japan, China, Malaysia, Indonesia, the Philippines, Israel, and many other Pacific Islands[10-12].

The clinical symptom of intermittent cutaneous migratory swelling may persist for many years. Diagnosis is currently based on a number of clinical criteria[9,13]. However, a few other parasitic infections may also cause migratory swelling that cannot be readily distinguished from that caused by *G. spinigerum*[14,15]. The host-parasite relationship in human gnathostomiasis, especially immune-responses, is not completely understood and the data of serological immunoassay have shown limited potential use. Many studies have demonstrated an increase in antibody titer that can, however, be used as evidence of recent infection and also of a strong likelihood of existing infection. This might be used to support a clinical diagnosis of gnathostomiasis [16-24]. However, like the immunodiagnosis of other parasitic infections, the specificity of the tests leave much to be improved. This is due to the antigen used of crude somatic aqueous extract[25].

Generally, the most common source of nematode immunodiagnostic antigen is the whole worm extract, but the glandular secretions (excretory-secretory products ; ES) are currently being considered. The development of suitable *in vitro* cultivation techniques for nematodes is essential for the practical preparation of acceptably pure antigens. In this regard, the cultivation system has been currently developed in many nematode serology [27]. It was desirable to use synthetic culture media with unsupplemented by host factors or other undefined components. Furthermore, it was necessary to simplify antigen purification as well as to permit antigen adsorption to immunoassay solid-phases without the competitive effects of other macromolecules. Characterization of nematode secreted products suggests that there are usually simple mixtures of relatively few components compared with the multiplicity of antigens present in crude whole worm preparation[26]. We have previously shown that *G. spinigerum* L_3s somatic extract comprised more than 29 polypeptides in polyacrylamide gel electrophoresis (SDS-PAGE)[28]. By immunoblotting analysis, four components which are strongly recognized by IgE antisera of gnathostoma patients have been identified. Of these, one antigenic band gives a consistent reaction with gnathostoma patient antisera. The specificity of this protein band is 99 per cent. The other protein bands are cross-reacted with IgE antibody from those with other intestinal parasitic infections. In addition, the ES products of this *G. spinigerum* L_3s have at least 4 bands in SDS-PAGE. A few protein bands show cross reaction with other intestinal parasitic infection, whereas, one band is specific with IgE antisera of this infection (unpublished observations). The previous studies have demonstrated that among the components released by the worm, major immunogens are present in ES material[26]. Infections with helminth and some arthropod parasites are characterized by elevated IgE antibody responses, particularly when mucous membrane or skin are involved at some stage of the parasite life cycle[3,5, 29]. This association has made scientists study the parasite allergens and detect the specific IgE-Ab responses. For the parasite allergen, much attention has been focused on ES products[3,27,30,31]. These antigens are more likely to be specific than the crude somatic or have a high potential for diagnositic evaluation especially for IgE-Ab detection [26,30]. On the other hand, some recent evidence has indicated that the parasite ES is also highly cross-reactive with other parasites[30-33]. These controversies leave much to be proved in gnathos-

tomiasis. The data of using ES products for IgE antibody detection of human gnathostomiasis have not been reported.

The aim of this study was to detect the IgE antibody in gnathostomiasis patients compared with patients with other parasitic infections by using crude somatic extract and ES products of *G. spinigerum* L_3s. The information of IgE antibody response may help to understand the humoral immune responses, thus leading to improved monitoring of the serodiagnosis and management of the patients.

MATERIAL AND METHOD

Specimens

Sera were obtained from patients attending the outpatient parasitology clinic of King Chulalongkorn Memorial Hospital, Bangkok, Thailand. Stool examinations for intestinal parasitic infections and complete blood count (CBC) were routinely performed in patients who had intermittent cutaneous migratory swelling (CMS) consistent with infection by *G. spinigerum*. Twenty two patients diagnosed with gnathostomiasis who had no intestinal parasites and had positive anti *G. spinigerum* L_3s IgG-antibody by Western blot analysis, were recruited for this study. Ten patients with parasitologically proven gnathostoma larvae recovered were also recruited. Healthy adults with no history of gnathostomiasis and negative for intestinal parasite served as the healthy controls. There were 73 additional patients with other intestinal parasites also available for the study.

Antigens preparation crude somatic aqueous extract

Third stage larvae of *G. spinigerum* were obtained by liver digestion of infected eels purchased from Klong-Toey market. The livers were pooled and digested at 37°C with 1.5 per cent pepsin pH 2.0 for 4 hours. *G. spinigerum* L_3s were individually picked and separated from the digested liver tissue under a dissecting microscope. Crude somatic aqueous extract of *G. spinigerum* L_3s was prepared by homogenization in a ground-glass tissue grinder followed by sonication as described previously[28]. The protein concentration of this L_3s somatic antigen was determined by Lowry's method[34].

Excretory-secretory products preparation

The third stage *G. spinigerum* larvae ES antigen was prepared from spent culture medium (BME, Gibco, USA) containing 100 u/ml penicillin

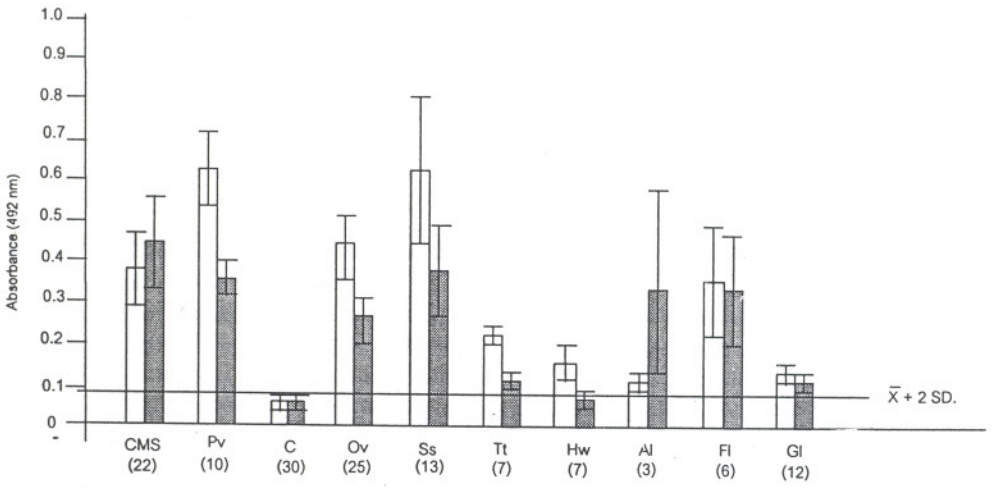

Fig. 1. **Optical density value (mean ± standard error of the mean) of the ELISA reaction for specific IgE antibody to somatic extract (□) and ES product (▨) in patient with intermittent cutaneous migratory swelling (CMS), in patient with proven *G. spinigerum* infection (Pv), compared to those of healthy control (C), Opisthrochis viverrini infection (Ov), strongyloides stercolaris infection (Ss), Trichuris trichiura infection (Tt), Hookworm infection (Hw), Ascaris lumbricoides infection (Al), Filariasis (FI) and Giardiasis (GI). The mean+ 2 standard deviation value for the normal control group (_____).**

Table 1. Comparison of ELISA value of using *G. spinigerum* third stage larva somatic extract and excretory-secretory (ES) products as antigens in gnathostomiasis and other parasitic infected patients.

Patients Group	Number tested	Absorbance density (492 nm)			
		Somatic Ag		ES Ag	
		Range	Mean ± SE	Range	Mean ± SE
Cutaneous migratory swelling	22	0.059 - 1.586	0.386 ± 0.082	0.100 - 1.435	0.464 ± 0.099
Proven gnathostomiasis	10	0.316 - 1.116	0.630 ± 0.097	0.132 - 0.664	0.378 ± 0.05
Healthy control	30	0.061 - 0.092	0.074 ± 0.001	0.071 - 0.095	0.079 ± 0.013
Opisthorchiasis	25	0.094 - 1.354	0.456 ± 0.100	0.085 - 0.907	0.272 ± 0.052
Strongyloidiasis	13	0.076 - 1.869	0.637 ± 0.167	0.090 - 1.236	0.395 ± 0.098
Trichuriasis	7	0.121 - 0.322	0.211 ± 0.033	0.082 - 0.132	0.109 ± 0.008
Paragonimiasis	7	0.109 - 0.304	0.165 ± 0.030	0.091 - 0.115	0.097 ± 0.003
Ascariasis	3	0.105 - 0.112	0.122 ± 0.013	0.093 - 0.117	0.350 ± 0.245
Filariasis	6	0.109 - 0.933	0.381 ± 0.130	0.113 - 0.982	0.370 ± 0.135
Giardiasis	12	0.071 - 0.310	0.140 ± 0.005	0.086 - 0.338	0.129 ± 0.005

Table 2 The number positive cases for anti *Gnathostoma spinigerum* third stage larva IgE antibody level in gnathostomiasis and other parasitic infected patients, using somatic extract and excretory-secretory (ES) products as antigens.

Patient groups	Number tested	Number positive			
		Somatic Ag		ES Ag	
			%		%
Cutaneous migratory swelling (CMS)	22	13	59.09	18	81.81
Proven gnathostomiasis	10	10	100	10	100
Healthy control	30	0	0	0	0
Opisthorchiasis	25	11	44.00	10	40.00
Strongyloidiasis	13	11	84.61	8	61.53
Trichuriasis	7	4	57.14	0	0
Paragonimiasis	7	1	14.28	0	0
Ascariasis	3	0	0	1	33.33
Filariasis	6	3	50.00	3	50.00
Giardiasis	12	2	16.66	1	8.33

G and 100 ug/ml streptomycin in which the worm had been maintained for 24 hours at 37°C under 5 per cent CO_2 in air as described previously(35). After incubation, the worms were removed and the collected spent medium was centrifuged at 10,000 g for 30 minutes at 4°C. The medium was concentrated by ultrafiltration using Amicon UM-2 membrane filter (Grace CO, USA). The concentrated spent medium was then dialyzed against distilled water containing proteinase inhibitors which were 0.1 mM phenyl-methylsulphonyl fluoride, 0.1 mM tosylamide –2- phenylethyl-chloromethyl ketone, 1 um N-(N-L-3 trans carboxyoxiran-2-carbonyl-L-leucyl) – agmatine. The protein concentration was determined by Lowry's method(34).

IgE antibody determination

Specific IgE antibody to *G. spinigerum* L_3s antigen was determined by enzyme-linked immunosorbent assay (ELISA). Wells of the microtiter plates (Immulon II ; Dynatech Laboratories, Inc., Alexandria, Va.) were coated with 50 µl of 5 µg/ml Ag. After incubation overnight at 4°C, the plates were washed with ELISA buffer. Serum (50 µl) with dilution of 1:5 was each added and allowed to react with the antigen at 37°C for 1 hour. Horseradish peroxidase-conjugated rabbit anti-human IgE (50 µl ; Dakopatts A/S, Copenhagen, Denmark) was then added. All conditions of conjugate dilution and IgE antibody titres for each specimen were optimized and calculated using the end point criteria as described by Soesatyo et al(24).

Table 3. Sensitivity and specificity of the ELISA for specific anti-*G. spinigerum* L_3s IgE antibody detection in patients with gnathostomiasis and other parasitic infected patients, using crude somatic extract and excretory-secretory (ES) products as antigens (Ag).

Antigen used	Results	Gnathostomiasis		Other parasitic patients	Sensitivity %	Specificity %	Predictive value	
		Suspected	Proven				Positive %	Negative %
Somatic Ag	+	13	10	31 (42.46%)	71.87	57.53	42.59	82.35
	-	9	0	42				
	Total	22	10	73				
ES Ag	+	18	10	22 (30.13%)	87.50	69.86	56.00	92.72
	-	4	0	51				
	Total	22	10	73				

RESULTS

ELISA values of anti *G.spinigerum* IgE antibody using 2 different antigens are presented in Fig. 1 and Tables 1-3. The levels of IgE antibody to gnathostoma antigens both somatic and ES products in 10 patients with parasitologically proven gnathostomiasis (Pv) were significantly higher than the background levels in the healthy controls ($P< 0.01$, Fig. 1). The mean ELISA values of these IgE antibody were also significantly higher in sera of suspected gnathostomiasis patients than in healthy controls ($P<0.01$). All of these suspected patients had been clinically diagnosed with cutaneous migratory swelling (CMS), IgG antibody positive to *G. spinigerum* infection by Western blot analysis and had a history of consuming improperly cooked fish. The ELISA values (optical density OD reading at 492 nm of a 1:5 serum dilution) of each group in this study were calculated by using Duncan's multiple range test. The IgE antibody levels for each specimen were calculated using the end point criteria as described by Soesatyo et al[24]. The sensitivity of the test using somatic antigen reduced from 100 per cent to 71.87 per cent when the suspected cases or CMS group were included (Table 3). By using ES antigen, the sensitivity was also reduced from 100 per cent to 87.50 per cent when the CMS group was included.

One hundred per cent negative and positive results were observed in the group of healthy controls and a group of proven cases (Table 2). The number of positive results in suspected patients or CMS group was not very high. These were 59.09 per cent and 81.81 per cent by using somatic extract and ES products (Table 2). However, when comparing somatic and ES antigens, the number of positives in the CMS group tended to be higher when ES antigen was used. This was statistically significantly different ($P<0.05$). In the group with other parasitoses, cross-reactivity was detected. The positive rate in each of these infections ranged from 0 per cent to 84.61 per cent (Table 2). The highest cross-reactive was found in strongyloidiasis. Somatic antigen showed a higher cross-reaction (42.46%) than the ES antigen (30.13%) but it was not significantly different (Table 3). Interestingly, the cross-reactive could not be found in trichuriasis and paragonimiasis when the ES antigen was used. For the specificity of the test, it was 100 per cent in the group of proven cases but decreased when the suspected cases were included. These showed 57.53 per cent and 69.86 per cent for somatic and ES antigen, respectively. Combining the data from parasitoses and healthy controls, the positive and negative predictive values were 42.59 per cent and 82.35 per cent. Both of these values of the test also tended to increase when ES products were used (56.00%, 92.72%, Table 3) but were not statistically significantly different.

DISCUSSION

Our results have clearly demonstrated that an infection caused by *G. spinig*erum could stimulate specific IgE antibody. Such antibody production in man and animals has been noted in many other helminth infections[35,36]. Nevertheless, the antibodies stimulation by this small migrating tissue larva have also been demonstrated in other isotypes including IgG antibody[16-23] and all the IgG subclasses (unpublished observations).

The worms which were surgically obtained or spontaneously emerged from the patients were

those of third-stage larvae or immature adults[12, 37]. The antigens used for the immunological test should be prepared from the stage associated with the disease. As such, this was the rationale in the selection of third-stage larvae for antigen preparation in the present study. Generally, the most common source of antigen is the whole worm extract. However, whole worm antigens have only a few components of functional antigens and hence, are potentially poor sources of specific antigens. For human gnathostomiasis, a number of recent reviews have demonstrated that the antigen of *G. spinigerum* somatic third stage larva extract has a high degree of cross reaction with other parasitic diseases including angiostrongyliasis[16,21], opisthorchiasis [20], paragonimiasis, taeniasis, and hook-worm infection[15,22]. In the present study, attention has turned to the use of antigen preparations which contain a more restricted range of antigen epitopes, particularly those obtained from parasite ES products. The ES products are usually simple mixtures of relatively few antigenic mosaic compared with the multiplicity of those present in crude somatic extract [18,36,38]. The previous report of Morakote et al showed that the ELISA values of anti-gnathostoma IgG-antibody had no significant difference for diagnosis of gnathostomiasis when comparing larval somatic with adult ES antigens[22]. Unfortunately, data of the third stage larva ES products were not reported, especially the anti-IgE antibody to this ES products.

In the present study, the levels of IgE-antibody to somatic and ES antigens in both group of CMS and the parasitologically proven group were significantly higher than those in the healthy control group ($P<0.01$). Comparing the use of somatic extract and ES products in the test, the positive rates in the group of suspected patients with gnathostomiasis or CMS were significantly higher using ES products (81.81%) as the antigens when compared with somatic extract (59.09%) ($P<0.05$, Table 3). The potential components may be present in greater proportion in ES products of the larva than those in somatic extract when the protein concentration is equal. However, the test was 100 per cent positive in the parasitologically proven group when both kinds of antigens were used. When approaching immunodiagnosis, most diseases are moving away from techniques based on precipitation and agglutination to the assay with, in general, higher sensitivity, such as ELISA. The sensitivity of our data was reduced from 100 per cent to the level of 71.87 per cent and 87.50 per cent by using somatic extract and ES products, respectively, when the suspected group was included. How to make an improvement may be not due to technical advances, but is more likely to come from selection of a suitable target antigen. Work is now in progress to identify and purify this antigen and to assess its value for diagnosis. By Western blot analysis, the specific component of *G. spinigerum* L_3s against IgE antibody is the peptide with a molecular weight of 43 kDa (unpublished observations). The cross-reactivity or the suboptimal specificity is primarily a consequence of sharing of antigenic epitopes among nematode parasites[36,40,41].

The diagnosis of suspected or CMS group in the present study was currently based on clinical manifestations, eosinophilia, positive IgG antibody to 24 kDa peptide in Western blot analysis and a history of consuming improperly cooked fish or other intermediate hosts. The specificity of the test was also reduced when the ELISA value of this group was included. These low levels of IgE antibody in the sera may be due to T cell immunoregulatory events. In addition, the IgE antibody can bind to the IgE receptors on the mast cell surface in the circulation and lose reaction with parasite antigen in the *in vitro* system. However, the reason for higher IgE antibody level in proven cases than that of suspected cases is still not known. The mechanism by which parasites exert potent stimulatory effect on the IgE antibody system in many nematode infections is under investigation in several laboratories[29,36,39,41]. It is possible that in proven gnathostomiasis, the larva has to come up and actively migrate in the cutaneous tissue and, then, in the skin for a period of time before immediately emerging from the skin[32]. During the emerging phase, the higher amount of exo-enzyme or allergen may have to be released. The principle of cutaneous or skin involvement is characterized by elevating the reaginic (IgE) antibody, particulary to a higher level compared to the deep tissue phase or the non-emerging phase[3,5,29,36,39]. Even the ES antigen produced some cross reaction in the ELISA of the present study, it can be used for further study in evaluation of the effectiveness of chemotherapy. Since the antibody responses to nematode-secreted products are more closely related to active

infection than the anti-whole worm antibody responses which may persist or even increase following death of the parasites(41). These ES products may be a suitable target antigen for identification and purification of the potential component(s). However, there is no information regarding the role of these specific IgE antibodies on the pathophysiology of this disease.

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การตอบสนองของ ไอจีอี แอนติบอดีย์ ต่อ โซมาติก และ อีเอส แอนติเจน จากตัวอ่อนพยาธิตัวจี๊ดระยะที่สามในผู้ป่วยโรคพยาธิตัวจี๊ด

วิไล ศักดิ์ศิริสัมพันธ์, วท.ม., รุ่งลาวรรณ เชวงเกียรติกุล, กศ.ม.**, กัญญรัตน์ กรัยวิเชียร, พ.บ.*, สุรางค์ นุชประยูร, พ.บ., ปร.ด.**

ศึกษาระดับ IgE แอนติบอดีย์ในซีรั่มของผู้ป่วยโรคพยาธิตัวจี๊ดในกลุ่มที่มีพยาธิตัวจี๊ดไชออกจากผิวหนัง และกลุ่มที่มีอาการปวด บวม เคลื่อนที่ของชั้นใต้ผิวหนัง (CMS) โดยวิธี ELISA ซึ่งใช้ somatic และ excretory–secretory (ES) แอนติเจนของตัวอ่อนพยาธิตัวจี๊ด *G. spinigerum* ระยะที่สาม ตัวอ่อนระยะที่สามนี้เตรียมขึ้นจากปลาไหลที่มีเชื้อโดยธรรมชาติ พบว่าผู้ป่วยทั้งสองกลุ่มมีระดับ IgE แอนติบอดีย์สูงกว่ากลุ่มคนปกติอย่างมีนัยสำคัญทางสถิติ ($P<0.01$) เมื่อเปรียบเทียบการใช้แอนติเจนทั้งสองชนิดนี้พบว่า ผู้ป่วยในกลุ่ม CMS ให้ผลบวก (81.81%) กับ ES แอนติเจน ซึ่งมากกว่า ผลบวกของ ELISA จากการใช้ somatic แอนติเจน (59.09%) ($P<0.05$) อย่างไรก็ตามทั้ง somatic และ ES แอนติเจน เกิดปฏิกิริยาข้ามกลุ่มกับซีรั่มของกลุ่มผู้ป่วยที่เป็นโรคพยาธิชนิดอื่น ๆ เมื่อวิเคราะห์ความไว (sensitivity) ของการทดสอบหา IgE แอนติบอดีย์นี้ พบว่าการใช้ somatic แอนติเจนให้ sensitivity 71.87 เปอร์เซ็นต์ และเพิ่มเป็น 87.50 เปอร์เซ็นต์ เมื่อใช้ ES แอนติเจน ความจำเพาะ (specificity) มีค่า 57.53 เปอร์เซ็นต์ เมื่อใช้ somatic แอนติเจนและเพิ่มเป็น 69.86 เปอร์เซ็นต์ เมื่อใช้ ES แอนติเจน ส่วนค่าการทำนายผลบวกและลบของการทดสอบนี้เท่ากับ 42.59 เปอร์เซ็นต์ และ 82.35 เปอร์เซ็นต์ เมื่อใช้ somatic แอนติเจน และค่าทั้งสองนี้เพิ่มขึ้นเป็น 56 เปอร์เซ็นต์ และ 92.72 เปอร์เซ็นต์ เมื่อใช้ ES แอนติเจน เป็นที่สังเกตว่า ES แอนติเจนน่าจะมีองค์ประกอบที่มีศักยภาพมากกว่า somatic แอนติเจน ซึ่ง ES แอนติเจนนี้ควรจะต้องนำมา purify ต่อและอาจเป็นแอนติเจนที่เหมาะสมสำหรับการประเมินประสิทธิภาพของการรักษา ทั้งนี้แอนติบอดีย์ที่ตอบสนองต่อสิ่งคัดหลั่งของพยาธิ น่าจะเกี่ยวข้องโดยตรงกับการติดเชื้อมากกว่าแอนติบอดีย์ที่มีต่อพยาธิทั้งตัว เพราะแอนติบอดีย์ชนิดหลังนี้อาจคงอยู่ แม้ว่าพยาธิถูกฆ่าหรือตายแล้ว อย่างไรก็ตาม ผลของ IgE แอนติบอดีย์ที่มีต่อ pathophysiology ของโรคนี้ก็ยังไม่เป็นที่เข้าใจกัน

คำสำคัญ : ไอจีอี แอนติบอดีย์, พยาธิตัวจี๊ด, โซเมติก แอนติเจน, อีเอส แอนติเจน

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